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(54) Title: CELL GROWTH REGULATORS

(57) Abstract

The invention relates to cytidine deaminase and its enhancers for use in inhibiting cell proliferation, especially haemopoiesis and to the use of cytidine deaminase inhibitors to stimulate cell proliferation, especially in leukopenic conditions and to mobilize stem cells to the blood.

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CELL GROWTH REGULATORS

5   The present invention is concerned with the control
of cell proliferation, especially the control of
haemopoiesis and/or granulopoiesis. In particular the
present invention is concerned with the use of cytidine
deaminase and/or its regulators to control cell
10   proliferation.

The mammalian body contains cells having enormously
diverse structures and functions, and the mechanisms of
differentiation and development have been the focus of
much study. It is known that for systems of cells
15   having a continuous turnover the mechanism commonly
involves a reservoir of pluripotent stem cells which
divide and constantly supply new cells to the system.
While initially homogeneous the stem cells supplied from
the "reservoir" soon become committed to one or other
20   morphology and subsequently develop into the required
functional cells.

Examples of such stem cell systems are the
haemopoietic system in bone marrow and the epithelial
and epidermal systems.

25   It is already known that mature granulocytes and
granulocyte extract (GRE) affect granulopoiesis (see
Rytömaa et al., p 106, "Control of cellular growth in
adult organisms" Academic Press (London), 1967 ed. Teir
and Rytömaa). Interestingly both stimulating and
30   inhibitory effects have been noted (see, for example,
Bøyum et al, Eur J. Haematol 38: 318 (1987) and
Helgestad et al, Acta Physiol Scand 133: 41 (1988)).
However, it has been suggested that the results obtained
in vitro depend upon the experimental design adopted

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(see Helgestad et al, supra).

Regulation of cell proliferation by stimulation or
inhibition continues to be of interest as a treatment
for diseases or conditions where natural control of cell
5   division has malfunctioned, for example in cancer (in
particular leukemia) or AIDS.

The enzyme cytidine deaminase (CDD) is responsible
for the conversion of cytosine to uracil in mammalian
cells. This reaction is also catalysed by other
10   enzymes, including deoxycytidylate deaminase and a
sequence specific cytidine deaminase involved in the
editing of apolipoprotein B mRNA.

CDD has been isolated from human tissues, and
sources include the liver, spleen and placenta (see Ho,
15   Cancer Res 33: 2816-2820 (1973) and Cacciamani et al,
Arch Biochem Biophys 290: 285 (1991)). Additionally,
Chabner et al. (J Clin Invest 53: 922-931 (1974))
reported the partial purification and characterisation
of CDD from normal and leukemic granulocytes. A highly
20   purified form of CDD (E.C. 3.5.4.5) has been isolated
from human placenta (see Laliberté et al, Cancer
Chemother Pharmacol 30: 7-11 (1992)).

CDD is known to be a 52 kD protein composed of four
subunits which are currently thought to be identical.
25   CDD continues to be of interest in the field of cancer
therapy since this enzyme is responsible for the
deamination of the widely used anti-cancer agent
cytidine arabinoside (Ara-C). The product of this
deamination reaction is uridine arabinoside (Ara-U)
30   which, besides being much less effective therapeutically
than Ara-C, is thought to cause neurotoxicity. Since in
Ara-C chemotherapy the active agent is continually
degraded by cytidine deaminase, high dosages of Ara-C
are often administered in order to maintain that

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compound at a therapeutically effective level in the body. However central nervous system toxicity including cerebellar dysfunction and peripheral neuropathy have been observed in patients treated with high dosages of

5   Ara-C. To minimise the amount of Ara-C required to maintain a therapeutically effective level, the CDD inhibitor tetrahydrouridine (THU) has been administered as part of the chemotherapy regime (see Kreis et al, Leukemia 5(11): 991-998 (1991)). THU acts by

10   competitively inhibiting CDD, thus reducing the amount of Ara-C which is deaminated by that enzyme. Other inhibitors of CDD such as Zebularine, 5-F-Zebularine and diazepinone riboside have also been suggested for use in combination chemotherapy with Ara-C or its analogue 5-

15   aza-2'-deoxycytidine (see Laliberté et al, Cancer Chemother Pharmacol 30: 7-11 (1992)). In each of the studies discussed above, administration of CDD inhibitors was only proposed as a supplement, to prevent degradation of the agent causing inhibition of cell

20   proliferation, namely Ara-C and 5-aza-2'-deoxycytidine.

It has now been found that cytidine deaminase (CDD) produced for example by mature granulocytes acts directly to inhibit the proliferation and colony formation of human and murine granulocyte-macrophage

25   progenitor cells (GM-CFC).

The present invention thus provides the enzyme cytidine deaminase (CDD) or a functional fragment thereof for use in the regulation of cell proliferation, particularly in the regulation of haemopoiesis, for

30   example in the inhibition of haematopoietic stem cell proliferation or of granulopoiesis. Thus, for example, it may be desirable to protect haematopoietic stem cells during anti-cancer chemotherapy or radiotherapy by interrupting their cell division cycle.

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Compositions comprising cytidine deaminase form a further aspect of the present invention.

Preferably, the cytidine deaminase is in an at least partially purified form. Substantially purified
5   cytidine deaminase is also preferred.

Generally, the effect of cytidine deaminase is inhibitory although the degree of inhibition depends on the concentration present. In particular, a reduced inhibitory effect has been observed at high
10   concentrations of CDD ie. a bell-shaped dose-response curve is obtained.

The cytidine deaminase used may be derived from any convenient source. Thus, for example, the enzyme may be isolated from bodily organs such as the liver or spleen
15   (see Ho (1973), Chabner (1974) and Cacciamani (1991) supra). More conveniently, however, the enzyme may be isolated from cell cultures - the cells either producing CDD naturally or as a result of transformation with recombinant DNA. In a particularly preferred embodiment
20   the enzyme CDD is produced from cells grown in culture which have been transformed or transfected with a DNA vector coding for cytidine deaminase, the cytidine deaminase gene being controlled by appropriate promoter and/or regulator sequences.

25   Harvesting and purification of cytidine deaminase may be by any suitable method. Suitable purification and separation techniques are well-known to those skilled in the art and include centrifugation, precipitation, dialysis, chromatography, including
30   affinity chromatography and column chromatography.

Human granulocytes contain large amounts of CDD, 9-10 times more than mononuclear cells. The amount drops to 36% of the normal value in the granulocytes with chronic myelogen leukemia. The reduction per cell is

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even greater with acute myelogen leukemia. Recent
findings indicate that the amount of CDD in normal and
leukemic cells can be approximately equivalent, however,
CDD activity is reduced in the leukemic cells.

5   The high number of leukemic cells can thus be
explained by a reduction of CDD or active peptide groups
from CDD. CDD may therefore be used in the treatment of
the symptoms.

         Thymidine is required as a co-factor for CDD as has
10   been previously observed for crude granulocyte extract
(see Helgestad et al, supra and Bøyum et al, Eur J.
Haematol 40: 119 (1988)). In agar cultures with human
granulocyte cells the inhibitory effect of CDD was
detectable with a thymidine concentration of 10^{-6}M.

15   Thus, the present invention also provides a
combination of cytidine deaminase or a functional
fragment thereof and one or more cofactors which enhance
the activity of cytidine deaminase, for use in the
regulation of cell growth, in particular haemopoiesis,
20   especially granulopoiesis, for example in chronic
myelogen leukaemia. Such cofactors include nucleosides
or analogues thereof, in particular pyrimidine
nucleosides and analogues thereof, especially those
selected from the list consisting of thymidine,
25   deoxycytidine, deoxyuridine and their phosphate
derivatives. Thymidine is especially preferred.
Conveniently the thymidine is present at a concentration
range of 5×10^{-3} to 1×10^{-6}M. The thymidine or other
cofactor may be administered simultaneously or
30   sequentially with the CDD.

         Thymidine is of course naturally present in the
body and it may therefore not be essential for thymidine
to be administered at all for inhibition of cell
division to be achieved.

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Ensminger et al (Cancer Res 37: 1857 (1977))
reported a median thymidine concentration of $0.2 \times 10^{-6}M$
in human plasma. Whilst this concentration appears
lower than that required *in vitro*, it is believed that
5   thymidine concentration at particular sites in the body,
for example in the bone marrow, may be locally elevated.
In the bone marrow for example large numbers of
erythroid nuclei are extruded from normoblasts and then
engulfed by macrophages in which DNA degradation takes
10   place, thus generating *inter alia* thymidine.

Thymidine has itself been reported to cause
inhibition of cell proliferation (see Blumenreich et al,
in Cancer Research 44: 2203-2207 (1984), Chiuten et al,
in Cancer Research 40: 818-822 (1980) and Leyva et al,
15   in J. Cancer Res. Clin. Oncol. 107: 211-216 (1984)).
Whilst relatively high dosages of thymidine (giving
millimolar concentrations in the plasma) did cause some
remission in cancer patients, it was not shown to be an
effective anti-cancer therapy and induced side-effects
20   such as nausea, vomiting and hepatotoxicity. The
inhibitory effect of thymidine on cell division was
believed to be due to allosteric inhibition of the
enzyme ribonucleotide reductase.

Whilst we do not wish to be bound by theoretical
25   considerations, several mechanisms can be put forward to
explain the inhibitory effect of CDD. One possibility
is that the combination of CDD and thymidine have a
synergistic effect on the depletion of the deoxycytidine
precursors required for DNA synthesis. Thymidine may
30   activate CDD directly or may neutralize an antagonist of
CDD. Previous studies have indicated that excess
thymidine (alone) may cause increased levels of
deoxythymidine triphosphate (dTTP) which is believed to
inhibit ribonucleotide reductase (see Reichard,

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Biochemistry 26: 3245 (1987) and O'Dwyer et al, Cancer
Res 47: 3911 (1987)). Inhibition of ribonucleotide
reductase would prevent or reduce the conversion of
cytidine to deoxycytidine. As indicated above CDD

5   deaminates deoxycytidine, again lowering the
concentration of this metabolite. Whilst thymidine and
CDD separately only have a marginal effect in isolation,
their combination could cause a significant deficiency
in deoxycytidine triphosphate (dCTP) (which is one of

10   the precursors of DNA) leading to failure of cells to
divide.

In an alternative hypothesis CDD binds to a
specific receptor on the cell surface, the binding or
subsequent steps in the mechanism being enhanced by the

15   co-factor thymidine. The binding of CDD to its receptor
may then cause transmission of a signal which prevents
or inhibits cell division.

Thymidine thus acts to enhance the effect of
cytidine deaminase and at higher concentrations, for

20   example 3-6 x 10^{-5}M, thymidine causes CDD to exhibit a
much stronger inhibitory effect ie. 50-90% suppression
of normal cellular proliferation. Other regulators
which enhance the CDD-mediated inhibition of cellular
proliferation include the nucleosides deoxycytidine and

25   deoxyuridine. Both deoxycytidine and deoxyuridine may
replace thymidine in enhancing the inhibitory action of
CDD. Additionally, a mixture of the nucleosides
deoxycytidine and thymidine act synergistically in
promoting the inhibition of cell division exhibited by

30   CDD. Thymidine phosphates such as thymidine
monophosphate and thymidine triphosphate act in the same
way as thymidine. These are inhibitors of deoxycytidine
monophosphate deaminase (dCMPD) and other inhibitors of
dCMPD such as 5-fluorodeoxyuridine (5-FdU) and 5-fluoro-

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deoxyuridine monophosphate (F-dUMP) act similarly.

CDD and its potentiators could be used in combination therapy with anti-cancer agents. By use of CDD and CDD potentiators, cells of interest, for example the bone marrow, could be taken out of cycle and thus be protected (ie. made less sensitive) to the effects of anti-cancer agents.

In our experiments thymidine was used at rather low concentrations, but nevertheless yielded 20-30% inhibition (p<0.01) ($4-8 \times 10^{-5}$ M thymidine) in combination with CDD in a granulocyte extract (GRE) from 1.6×10^5 granulocytes (/ml). At the high GRE concentration (10^6 cells/ml) thymidine (8×10^{-5}M) yielded no inhibition. FdUMP had itself an antiproliferative effect at the highest concentrations, but this effect was partly abolished when combined with thymidine. The most striking effect of FdUMP was the ability to induce inhibition also together with a high GRE concentration.

In further experiments, mononuclear human blood cells were cultured in fetal calf serum or serum from the same individuals (autologous serum), at two different thymidine concentrations. In comparison with fetal calf serum cultures there was a striking effect on colony formation by human serum in cultures with 3.3×10^{-5} M thymidine, the colony number was reduced by 50%, and with 16×10^{-5} M a 90% reduction was observed. The effect of thymidine was to a large extent prevented by adding THU to the cultures. The thymidine increase had no effect on colony formation in fetal calf serum cultures but addition of GRE (containing CDD) caused a strong inhibition, which could be abrogated by THU. It thus appears that human serum contains CDD that induces inhibition of colony formation when thymidine is added. Accordingly it was shown by thin layer chromatography

that CDD activity could easily be detected in fresh
human serum. No appreciable activity was found in fetal
calf serum. However these sera have been subjected to
freezing/thawing which tends to reduce CDD activity. In

5   any case, these finding may suggest the CDD plays a
physiological role in regulation of white cell
production.

It follows from the above considerations that THU
may be used in the treatment of some diseases where

10   there is a need for stimulating cell proliferation by
inactivating CDD. Furthermore thymidine and other CDD
enhancers may be used in certain diseases to enhance the
inhibitory effect of CDD in tissue fluid. Alternatively
thymidine and CDD may be used in combination. Large

15   doses of thymidine have been used in the treatment of
malignant diseases, with little success. However, for a
disease like chronic granulocytic leukemia, thymidine
dosage may enhance the inhibitory effect of CDD whereby
production of granulocytes may be maintained at a lower

20   level.

The invention thus provides CDD potentiating
nucleosides or analogues thereof for use in the
regulation of cell proliferation mediated by cytidine
deaminase, for example chronic myelogen leukaemia.

25   Thymidine, deoxycytidine and deoxyuridine are examples
of suitable nucleosides. They may be used in the form
of their phosphate derivatives. Thymidine or a
combination of thymidine and deoxycytidine are
preferred. The nucleosides or analogues thereof are

30   conveniently added to give concentrations of 10^{-6} to
10^{-4}M, for example 5×10^{-4} to 5×10^{-5}M.

Inhibitors of CDD itself, for example the inhibitor
THU discussed above, may also be used according to the
invention to control or prevent the inhibition of cell

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proliferation. In particular, inhibition of CDD may be
used to control or prevent the inhibition of cell
proliferation due to CDD. Other inhibitors of CDD which
affect cellular proliferation in the same way include

5   Zebulaire, 5-F-Zebulaire, 5-chloromercuricytidine, CV6,
2-azido-2-deoxycytidine, 5,6-didehydro-uridine and
diazepinone riboside. Additionally antibodies to CDD
may also be used as CDD inhibitors where these bind to
or interfere with the active site of CDD. As discussed

10   above, the use of THU with the anti-leukemic drug Ara-C
has already been investigated (see Kreis et al, supra).
Whilst the action of THU in competitively inhibiting CDD
was appreciated in that study, THU was administered
simply to prevent the undesirable deamination of the

15   drug of interest (Ara-C) by CDD. It was, however, not
appreciated that CDD affected cell proliferation itself.

CDD inhibitors may be of use in the regulation of
leukopenic situations in general and more particularly
after anti-cancer treatment or bone marrow

20   transplantations or in connection with the treatment of
infections. CDD inhibitors are believed to increase the
number of white blood cells and/or their activity.

It will be appreciated that dCMPD referred to above
is antagonist of CDD and thus acts as a CDD inhibitor.

25   In a further aspect, the present invention thus
provides the use of CDD inhibitors in the regulation of
cell proliferation, in particular cell proliferation
mediated by CDD. Another aspect of the invention
provides the use of CDD inhibitors, such as THU, in the

30   manufacture of a medicament for the regulation of cell
proliferation, in particular cell proliferation mediated
by CDD. In a still further aspect, the present
invention provides the use of one or more CDD inhibitors
in the preparation of a medicament for the mobilization

of haematopoietic stem cells to the blood.

In a further aspect, the present invention provides
a pharmaceutical composition comprising (a) cytidine
deaminase or a functional fragment thereof optionally in
5   combination with a CDD potentiating nucleoside or
analogue thereof or (b) an inhibitor of cytidine
deaminase.

In the pharmaceutical compositions according to the
present invention the usual pharmaceutically acceptable
10   inert carriers, diluents, additives, flavourings and/or
colourings may of course be present as required.
Suitable adjuvants and excipients will be known to those
skilled in the art.

In a still further aspect, the present invention
15   provides a method of treatment of the human or non-human
animal body to regulate cell proliferation, said method
comprising administering to said body cytidine deaminase
or a functional fragment thereof. The cytidine
deaminase or functional fragment thereof may also be
20   used in combination with one or more cofactors which
enhance the activity of cytidine deaminase. A further
method of treatment to regulate cell proliferation which
is provided by the present invention comprises
administration of one or more cytidine deaminase
25   inhibitors.

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In the diagrams:

Fig. 1   illustrates the effect of different
             concentrations of deoxycytidine, alone or in
5   combination with thymidine in inhibiting
             colony formation in the presence of
             granulocyte extract (GRE);

Fig. 2   shows how colony formation is affected by
10   addition of GRE, with and without thymidine or
             deoxyuridine;

Fig. 3   depicts the relationship between colony number
             and dose of GRE at different concentrations of
15   thymidine;

Fig. 4   shows the inhibition of colony formation at
             varying concentrations for different Mono Q
             fractions of GRE;
20
Fig. 5   is a bar chart relating to the dose of GRE to
             colony number, optionally including Ara-C at
             concentrations of 10^{-7}M and 10^{-5}M;

25 Fig. 6   is a bar chart showing how colony number is
             affected by the concentration of azacytidine
             with and without GRE;

Fig. 7   illustrates how colonies of mouse BMC and
30   human blood cells treated with GRE and
             affected at different concentrations of THU:

Fig. 8   shows the inhibition of colony formation and
             CDD activity (measured by deoxyuridine

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concentration) for different fractions of GRE
following Mono Q;

Fig. 9   illustrates the results of MTT-assay for
different Mono Q fractions of GRE; and

Fig. 10   shows the results of MTT-assay for fraction 5
(of the Mono Q purification of GRE) at
different concentrations.

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The present invention is further illustrated by the following, non-limiting, examples.

Materials and Methods

Chemicals: Thymidine, cytidine, deoxycytidine, deoxyuridine, 5-azacytidine, 5-aza-2'-deoxycytidine, and cytosine arabinoside were obtained from Sigma. Tetrahydrouridine was obtained from Calbiochemic (La Jolla, Ca). Deoxy[5-^{3}H]-cytidine, specific activity 1.07 TBq/mmol, was obtained from Amersham. McCoy's 5A medium, RPMI 1640 and CMRL 1066 medium were obtained from Flow. The CMRL 1066 medium was used with additives (Helgestad et al, supra). Lymphoprep was provided by Nycomed AS, Oslo. Murine interleukin 3 was obtained from Genzyme (Cambridge, Ma) and murine granulocyte-macrophage colony-stimulating factor (GM-CSF) from Pepro Tech Inc. (Rocky Hill, NJ).

Cells: Bone marrow cells (BMC) were obtained from the femurs of female B_6D_2 mice (Bomholdt gaard, Denmark). Mononuclear cells and granulocytes from human blood and buffy coat samples were separated with Lymphoprep (Bøyum et al, Scand J. Immuno 34: 697 (1991)). A slow centrifugation (60g, 10 min) was included to remove platelets from the mononuclear cells. Contaminating erythrocytes in the granulocyte fractions were lysed by incubating the cells in 0.83% NH$_4$Cl for 7 minutes at room temperature. After centrifugation (600 g, 7 min), the supernatant was removed, the cells resuspended in 0.9% NaCl and counted. NFS 60 cells, an early murine myeloid cell line, the C6 cell line, a fibroblast type murine cell, and a human bladder carcinoma cell line (5637) were kindly provided by Dr. Andrew King, SmithKline

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Beechham (Pa, USA). These cells, and mouse L-cells
(NCTC Clone 929), were cultured in RPMI 1640 medium with
10% FCS, and were subcultured once or twice weekly. The
supernatant from L-cells was used as source of

5   macrophage CSF. The medium conditioned by the carcinoma
cell line (CM 5637) is a rich source of human G-CSF
(Welte et al, PNAS 82: 1526 (1985)). The NFS 60 cells
were cultured in the presence of 2% (v/v) CM 5637.

10   Granulocyte extract (GRE): The granulocyte pellet
obtained after NH_4Cl treatment and washing was suspended
in water for 4-5 minutes, at a concentration of 200 x 10^6
cells/ml. The supernatant was collected after
centrifugation and stored at -20°C until used.

15

GM-CFC assay: Mouse BMC (5 x 10^4 per plate) were
cultured in 0.3% agar (Bacto-agar, Difco) in CMRL 1066
medium and 16% fetal calf serum. CM 5637 was used (0.1
ml per 1 ml culture plate) as stimulator. After 7 days

20   of incubation at 37°C and with 7.5% CO_2 in humidified
air, the colonies (>50 cells) were counted. Mononuclear
human blood cells (5 x 10^5/ml) were cultured for 14 days
in 0.3% agar, in McCoys's 5A or CMRL 1066 medum, with
16% FCS. No stimulator was added. Aggregates of more

25   than 40 cells were counted as colonies. The cultures
were run in triplicate.

MTT-assay: Proliferation of established cell lines was
measured by a colorimetric method (Mosmann, J Immunol

30   Meth 65: 55 (1983)). The cells were seeded in
microtitre plates (Costar 3596, Cambridge, Mass.) at a
concentration of 2 x 10^4 cells/well in 100 μl of CMRL
1066 medium with 15 x 10^{-5}M thymidine and 5% FCS
(Hyclone, Logan, Utah). Fifty μl of test samples in

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various dilutions was added to each well. After
incubation for 4 days in a humidified atmosphere of 5%
CO_2 in air at 37°C, 15 µl of MTT (3-(4,5-dimethylthiazol-
2-yl)-2,5-diphenyltetrazolium bromide; Tiazolyl Blue)
5   (Sigma) at a concentration of 5 mg/ml in phosphate
buffered saline was added, and the cultures were further
incubated for 3 hours. Thereafter 100 µl of isopropanol
was added to all wells. The microtiter plates were
shaken at room temperature for 30 minutes and then the
10   remaining dark blue crystals were dissolved by
pipetting. The optical densities of the wells were read
on a Dynatec MR 700 microplate reader with a test
wavelength of 570 nm and a reference wavelength of 630
nm.
15

Cytidine deaminase assays: Reaction mixtures contained
50 mM Hepes, pH 7.5; 0.1 mM deoxy[5-³H]-cytidine
(specific activity 18.5 MBq/mmol); and protein fractions
to a final volume of 5 µl, and was incubated for 30
20   minutes at 37°C. The reaction was stopped by addition
of unlabelled deoxycytidine and deoxyuridine in
equimolar amounts (1 µl of 5 mM each) followed by
application on a Polyethyleneimine thin layer sheet
(plastic backing, Schleicher & Schüll). The
25   chromatograms were developed in isopropanol/0.1M HCl
(7:2) which gives Rf values of 0.73 for deoxyuridine and
0.51 for deoxycytidine, respectively. Spots containing
deoxyuridine and deoxycytidine were visualized by UV
(254 nm), and radioactivity in each spot determined by
30   scintillation counting.

Protein chromatography: GRE (4 ml, approximately 40 mg
protein) was diluted with 6 ml buffer A (50 mM NaCl; 50
mM Hepes, pH 7.5; 1 mM EDTA) and applied to a DEAE-

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Sephacel (Pharmacia) column (1.3 x 2.6 cm)
preequilibrated with buffer A. The column was washed
with 10 ml of buffer A and eluted with 6 ml of 400 mM
NaCl (50 M Hepes, 1 mM EDTA). The eluate was subjected

5   to buffer exchange to buffer A with a PD-10 column
(Pharmacia), and applied to a Mono Q anion exchange
column (0.7 x 5.5 cm, FPLC system, Pharmacia). The
column was washed with 20 ml of buffer A and eluted with
a linear gradient to 1000 mM NaCl at a flow rate of

10   0.25 ml/min. Fractions (1 ml) were sterile filtered and
tested for inhibitory activity in the GM-CFC assay or
for cytidine deaminase activity as indicated above.
Active fractions were further purified by gel filtration
(1.5 x 25 cm Ultrogel AcA 34) and eluted with buffer B

15   (400 mM KCl; 50 mM Hepes, pH 7.5; 1 mM EDTA, 25%
glycerol, 1 mM mercaptoethanol). Fractions (1 ml) were
sterile filtered and assayed for GM-CFC inhibitor and
cytidine deaminase activity as described above. The gel
column was precalibrated with myoglobin (17.5 kD),

20   ovalbumin (47 kD), and blue dextran (reflecting void
volume) under identical flow conditions.

Statistics: Students t-test or Wilcoxon's non-
parametric test was used for statistical calculations.

25

EXAMPLE 1

Inhibition of granulocyte-macrophage colony-forming
cells (GM-CFC) from human blood

30

The separate and combined effect of thymidine (3 x 10^{-5}
M) and deoxycytidine on the capacity of extract (GRE)
from 10^6 granulocytes to inhibit growth of granulocyte-
macrophage colony forming cells (GM-CFC) from human

blood were investigated. Mononuclear human blood cells
(5 x 10^5) were cultured in McCoy's medium in agar, and
the colonies were counted on day 14. Control cultures
contained an average of 30 (18-58) colonies. Mean

5   values (± SD) from 5 experiments are given as a
percentage of control value. The cultures were run in
triplicate in each experiment. Thymidine or
deoxycytidine alone had no apparent effect on colony
number. The results are shown in Figure 1.

10

As illustrated in Figure 1 thymidine and deoxycytidine
had an additive effect (p<0.05). It is also shown that
deoxycytidine could replace thymidine as a co-factor for
the granulocyte extract at a concentration of 2 x 10^{-4}M

15   ie. at a 10x higher concentration than the concentration
of thymidine required to produce strong inhibition.

EXAMPLE 2

20   Inhibition of GM-CFC by thymidine or deoxyuridine with
and without granulocyte extract

Human blood GM-CFC were grown in agar cultures.
Thymidine or deoxyuridine, alone or with 10^6 GRE

25   (granulocyte extract) were added in McCoy's medium. The
inhibitory effect on GM-CFC was observed. McCoy's
medium is nucleoside free. A comparison with CMRL 1066
medium (contains 4.0 x 10^{-5}M thymidine) was also
performed.

30

The results, shown in Figure 2, are the mean values (±
SD) from 3 experiments. Figure 2 shows that
deoxyuridine can replace thymidine as a co-factor for
the granulocyte extract at an approximately 10 fold

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higher concentration than thymidine to achieve the same
cellular inhibition (5 x 10^{-4}M of deoxyuridine required
compared to 10^{-5}M thymidine).

5   High concentrations (1-5 x 1^{-4}M) of deoxyuridine showed
no suppressive affect on GM-CFC. In contrast, the
lowest deoxyuridine concentration (10^{-4}) enhanced colony
formation (p<0.01).

10   On the other hand, thymidine at high concentration (5 x
10^{-4}M) exerted an inhibitory effect in the absence of
granulocyte extract.

EXAMPLE 3

15

Inhibition of Mouse Bone Marrow cells by GRE

Mouse bone marrow cells (BMC) were cultured for 7 days
in CMRL 1066 medium in agar. The supernatant from
20   cultures of the 5637 cell line was used as stimulator.
Granulocyte extract was added in increasing doses. Mean
values (±SD) were obtained from 3 replicate cultures.

Mouse bone marrow cells have previously been shown to be
25   sensitive to GRE and thymidine (See Bøyum et al, Eur J.
Haematol 40: 119 (1988)). Here, it was found that 4 x
10^{-5}M thymidine was sufficient to cause strong
(approximately 80%) inhibition of cells cultured in
methylcellulose.

30

To obtain the same degree of inhibition in agar culture,
it was found in a comparative study that the thymidine
concentration had to be increased by 3-4 fold. The
results shown in Figure 3 indicate that strong

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inhibition of murine GM-CFC in agar required a thymidine
concentration of 13×10^{-5}M.

EXAMPLE 4

The effect of GRE on colony formation induced by
different stimulators

The low density fraction of mouse BMC separated with
Lymphoprep were cultured in McCoy's medium with 13×10^{-5}M
thymidine. Recombinant IL-3 (100 units/ml) rGM-CSF (10
ng/ml), L-CSF (0.1 ml/plate) and CM 5637 (0.1 ml/plate) were
used as stimulators. Mean values (± SD) from 2 separate
experiments.

Table 1

Stimu-lator	Colony number in control per 5×10^4 BMC	Colony number as a percentage of control value		
		GRE	Thymidine	Thymidine + GRE
IL-3	163±27	86±3	89±3	11±6
GM-CSP	108±16	95±6	115±5	60±7
L-CSF	222±17	100±9	108±10	52±7
CM 5637	262±36	101±6	92±5	30±3

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EXAMPLE 5

Isolation of the fraction of GRE with GM-CFC inhibitory
activity

Four ml GRE (from 800x10⁶ cells, approximately 40 mg
protein) was separated with DEAE-Sephacel and anionic
exchange chromatography (Mono Q, see "Materials and
Methods") and tested for inhibitory activity on GM-CFC
in mouse bone marrow cultured in CMRL 1066 medium in
agar with 13×10^{-5}M thymidine. Fraction 1 indicates the
start of elution with 1000 mM NaCl. The elution was
completed in 30 minutes. The mean values (± SD) from 10
separations are given in Figure 4.

After anionic exchange chromatography, it was observed
that the inhibitory activity of fractions 4 and 5 was
lower at the highest concentration (dilution of fraction
by 1/50). The phenomenon was not observed for fraction
6, but has been previously reported for crude
granulocyte extract (see Bøyum et al (1980) supra).

EXAMPLE 6

Prevention of Inhibitory Action of Cytosine Arabinoside
(Ara-C) and Azacytidine by GRE

GM-CFC from human blood were cultivated and the
inhibitory effects of GRE, Ara-C and GRE with Ara-C were
investigated. The results are shown in Figure 5.

Parallel experiments were performed using azacytidine
instead of Ara-C and the results are given in Figure 6.

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At concentrations of $10^{-7}M$, Ara-C caused almost complete
suppression of cell division in GM-CFC. This
suppression was alleviated by addition of extract from
10^5 granulocytes. Control experiments showed that GRE
5   from the same batch suppressed GM-CFC in thymidine
supplemented cultures (CMRL 1066).

Similarly, crude GRE prevented the inhibitory effect of
azacytidine - see Figure 6, (p<0.01).
10

EXAMPLE 7

The effect of tetrahydrouridine (THU) on GRE induced
suppression of colony formation by mouse bone marrow
15   cells and human blood cells

The cells were cultured in agar in CMRL 1066 medium with
either $3.3 \times 10^{-5}M$ (human cells) or $13 \times 10^{-5}M$ (mouse
cells) thymidine. GRE from 2×10^5 cells were added per
20   culture plate.

Figure 7 gives the results with mean values (±SD) from 4
mouse cell experiments and 3 human cell experiments.

25   The inhibitory effect of crude GRE on human and murine
GM-CFC was completely abolished by $4 \times 10^{-6}M$ THU. THU is
known to be a specific inhibitor of CDD and these
results therefore indicate that CDD is responsible for
the inhibitory effect observed. THU itself had no
30   noticeable effect on colony formation.

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EXAMPLE 8

Investigation of CDD activity in GRE fraction

5   The presence of CDD in GRE was investigated directly, by
testing for the ability to convert deoxycytidine to
deoxyuridine. The active fractions of the Mono Q
separation and separation (see Example 5) were further
separated by gel filtration. Figure 8 shows the elution

10   profile after gel filtration of cytidine deaminase and
inhibitory activity in GRE. Inhibitory activity on
mouse GM-CFC was measured in the same fractions. The
most active fractions were reexamined for CDD and
inhibitory activity, with almost identical results.

15   Closed symbols denote experiments with tetrahydrouridine
(THU). The elution positions of molecular weight
standards are indicated.

The growth inhibitor and CDD activity were found to

20   copurify, both eluting in a single peak of M_r 50 kD which
corresponds to the molecular weight of human CDD
reported earlier by Cacciamani et al in Arch Biochem
Biophys 290: 285 (1991).

25   THU at a concentration of 4×10^{-5}M neutralized the
inhibitory and deaminating ability in all active
fractions.

EXAMPLE 9

30

Inhibition of mouse GM-CFC, NFS 60 and C6 by the Mono Q
fractions of GRE

NFS 60 and C6 cells were cultured in microtitre plates

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(20,000 cells/well) for four days and the cell growth
was measured with a colorimetric method (MTT). GM-CFC
was monitored with the agar colony assay. Fractions
obtained after anionic exchange chromatography (Mono Q)
5   were added to NFS 60 and GM-CFC cultures after 800 fold
final dilution in growth medium and to the C6 cells
after 50 fold dilution. Figures 9 gives the results
from the mean (±SD) of triplicate cultures from one
representative experiment. The results were confirmed
10   twice, in one experiment with a different set of GRE
fractions.

It was found that the inhibitory activity for colony
formation affected murine GM-CFC and myeloid cell line
15   NFS 60 to a similar extent. Inhibition of colony
formation for the NFS 60 cell line was abolished by THU
addition (results not shown).

In contrast, the proliferation of a fibroblast cell
20   line, C6, was not suppressed by the GRE fractions,
indicating that there may be some cell specificity for
CDD.

EXAMPLE 10

25

Dose-Response Curve for Fraction 5 from Mono Q
purification

The most active fraction from Example 9 (Fraction 5 -
30   see Figure 9) was tested at several concentrations for
its ability to inhibit cell replication for murine GM-
CFC, NFS 60 and C6 cells. NFS 60 cells and C6 cells
were cultured in microtiter plates (20,000 cells/well)
for four days and the cell growth was measured with the
35   MTT assay. GM-CFC was monitored with the agar colony

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assay. Figure 10 gives the results of mean values (±
SD) of triplicate cultures from one representative
experiment. The results were confirmed in two
additional experiments.

Figure 10 shows the dose-response curve for fraction 5
at different dilutions. The inhibitory effect on GM-CFC
and NFS 60 cells was partially alleviated at high
concentrations of Fraction 5 GRE, thus giving a bell-
shaped response curve.

EXAMPLE 11

Bone marrow cells were cultured for 7 days in McCoy's
medium and then the colonies were counted.
Two granulocyte extracts were prepared, the first from
1.6×10^5 granulocytes per ml and the second from 8×10^6
granulocytes per ml.

The effect of thymidine and/or 5-fluoro-2-deoxyuridine-
monophosphate (FdUMP) alone or together with one of the
two granulocyte extracts on proliferation of the
colonies was tested. The results are shown in Table 2.
When thymidine and FdUMP were combined, the
concentration of thymidine was kept constant at 0.8×10^{-4}
M and FdUMP concentration was varied as shown in Table
2.

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<u>Table 2</u>

The effect of thymidine (T), 5-fluoro-2-deoxyuridine-
monophosphate (FdUMP) and T+FdUMP on the inhibitory
5   effect of granulocyte extract (GRE) on granulocyte/
macrophage colony formation.

Colony number as a percentage of control

$Conc^n$ T or FdUMP (M)	T	T + GRE 1	T + GRE 2	FdUMP	FdUMP + GRE 1	FdUMP + GRE 2	T + FdUMP	T + FdUMP + GRE 1	T + FdUMP + GRE 2
0	95	102	90						
0.8×10^{-4}	108	79	91	7	0	0	64	0	0
4×10^{-5}	109	82	94	50	0	0	76	0	0
0.8×10^{-5}	102	95	95	102	59	0	104	70	1
0.8×10^{-6}	109	93	80	115	94	63	95	84	62
0.8×10^{-7}	104	94	96	100	85	64	107	64	58

GRE 1 = extract from 1.6×10^5 granulocytes/ml. GRE 2 =
extract from 8×10^6 granulocytes/ml. When T and FdUMP
were combined, the concentration of T was kept constant
(0.8×10^{-4} M), and FdUMP concentration was varied as
5   indicated in the left column.

Despite the low concentration of $4-8 \times 10^{-5}$ M, thymidine
caused 20-30% inhibition ($p < 0.01$) with GRE 1. With the
higher concentration of GRE (GRE 2), thymidine caused
10   less inhibition. FdUMP itself had an antiproliferative
effect at high concentrations, but this effect was

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abolished when thymidine was also present.

FdUMP together with GRE 2 (high granulocyte extract concentration) caused significant inhibition of cell
5   proliferation.

EXAMPLE 12

Mononuclear human blood cells were cultured in fetal
10   calf serum (FCS) or autologous serum from the donor of
the blood cells. The cells were cultured at two
different thymidine concentrations (3.3×10^{-5} M thymidine
or 16×10^{-5} thymidine). Aggregates with more than 40
cells were counted as colonies. THU and/or GRE were
15   added and the effect on colony number was evaluated as a
percentage of the control values.

The cultures were run in triplicate and Table 3 shows
mean values (± SD) from two experiments. The mean
20   colony number in FCS cultures used as control was 259 ±
61.

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<u>Table 3</u>

Colony formation by human blood cells in cultures with
fetal calf serum (FCS) or autologous serum. The effect
of thymidine, granulocyte extract (GRE) and
tetrahydrouridine (THU).

Colony number as a percentage of control values with FCS
and low thymidine concentration.

Thymidine Conc. (M)	Serum	Controls	THU	GRE	GRE + THU
3.3×10^{-5}	FCS	100 ± 6	100 ± 6	34 ± 7	99 ± 7
3.3×10^{-5}	aut.ser	47 ± 6	81 ± 13	28 ± 2	73 ± 12
16×10^{-5}	FCS	103 ± 5	96 ± 7	16 ± 7	92 ± 9
16×10^{-5}	aut.ser	11 ± 3	63 ± 8	7 ± 4	77 ± 11

The higher thymidine concentration had no effect on FCS
cultures. In both culture types addition of GRE from 2
x 10^5 cells per plate caused strong inhibition on colony
formation. This was, however reversed by addition of
THU.

EXAMPLE 13

The effect of tetrahydrouridine (THU) on haemopoietic
progenitor cells in the blood was examined <u>in vivo</u>, in
mice.

A dose of 0.6 mg per mouse (strain DBA) was injected
intraperitoneally at time -4 hours (THUx1) or time -20
and -4 hours (THUx2), and blood was collected by heart
puncture at time 0 hours. White blood cell number was
determined and 1x10^5 cells were seeded in 1 ml 0.33% agar
in CMRL 1066 medium and incubated at 37°C at 7.5% CO_2 in

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air for the indicated time period. The results are
shown in Table 4.

<u>Table 4</u>

Effect of THU on colony number obtained from 1×10^5 blood
cells cultured in agar

	Day 7			Day 10			Day 13		
Exp. No.	Contr	THUx1	THUx2	Contr	THUx1	THUx2	Contr	THUx1	THUx2
G39	0.2		0.5	9.5		30.3	728		1847
G43	9.8	29.8	56.7	215	231	753	488	694	901
G48	0	0.3	0.2	14.2	238	227	96	806	852
G52	2.8	10.2	8.8	38.5	501	535	1166	1674	1609

An increase in the number of colonies from seeded white
blood cells was observed in blood taken from mice
treated with THU compared to control animals. THU thus
appears to increase the number of haematopoietic
progenitor cells in blood.

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CLAIMS:

1.   A compound selected from cytidine deaminase and a
functional fragment thereof for use in the regulation of
5   cell proliferation.

2.   The use of cytidine deaminase or a functional
fragment thereof for the manufacture of a medicament for
the regulation of cell proliferation.
10

3.   The compound or use according to claim 1 or 2
wherein the cytidine deaminase or a functional fragment
thereof is for use in combination with one or more
cofactors which enhance the activity of cytidine
15 deaminase.

4.   The compound or use according to any one of claims 1
to 3 wherein haemopoiesis is inhibited.

20 5.   The compound or use according to claim 4 wherein
granulopoiesis is inhibited.

6.   The compound or use according to claim 5 wherein the
cell proliferation in chronic myelogen leukaemia is
25 inhibited.

7.   The compound or use according to any one of claims 3
to 6 wherein the cofactors are cytidine deaminase
potentiating nucleosides or analogues thereof.
30

8.   The compound or use according to claim 7 wherein the
potentiating nucleosides or analogues thereof are
selected from the group consisting of pyrimidine
nucleosides or analogues thereof.
35

9.   The compound or use according to claim 8 wherein the
pyrimidine nucleosides or analogues thereof are selected

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from the group consisting of thymidine, deoxycytidine, deoxyuridine and their phosphate derivatives.

10.   The compound or use according to claim 9 wherein the nucleoside is thymidine.

11.   The compound or use according to claim 10 wherein thymidine is present at a concentration in the range 5×10^{-3} to 1×10^{-6}M.

12.   A composition comprising a combination of cytidine deaminase or a functional fragment thereof and one or more cofactors which enhance the activity of cytidine deaminase for use in the regulation of cell proliferation.

13.   The use of one or more cytidine deaminase potentiating nucleosides or analogues thereof for the manufacture of a medicament for the regulation of cell proliferation mediated by cytidine deaminase.

14.   The use according to claim 13 wherein the potentiating nucleosides or analogues thereof are selected from the group consisting of thymidine, deoxycytidine, deoxyuridine and their phosphate derivatives.

15. The use according to claim 14 wherein thymidine and deoxycytidine are used.

16.   The use according to any one of claims 13 to 15 wherein nucleosides or analogues thereof are added to give concentrations thereof of 10^{-4} to 10^{-6}M.

17.   The use according to claim 16 wherein said concentrations are in the range 5×10^{-4} to 5×10^{-5}M.

18.   The use of one or more cytidine deaminase
inhibitors in the preparation of a medicament for the
regulation of cell proliferation.

5   19.   The use according to claim 18 wherein the cell
proliferation is mediated by cytidine deaminase.

20.   The use of one or more cytidine deaminase
inhibitors in the preparation of a medicament for the
10   mobilization of haematopoietic stem cells to the blood.

21.   The use according to any one of claims 18 to 20
wherein the inhibitor is tetrahydrouridine (THU).

15   22.   The use according to any one of claims 18 to 21
wherein cell proliferation is stimulated in leukopenic
situations such as after bone marrow transplantation or
after chemotherapy.

20   23.   A pharmaceutical composition comprising (a)
cytidine deaminase or a functional fragment thereof
optionally in combination with a cytidine deaminase
potentiating nucleoside or analogue thereof or (b) an
inhibitor of cytidine deaminase, together with at least
25   one physiologically acceptable carrier or excipient.

24.   A method of treatment of the human or non-human
animal body to regulate cell proliferation, said method
comprising administering to said body cytidine deaminase
30   or a functional fragment thereof.

25.   A method of treatment according to claim 24, said
method comprising administering to said body in
combination with the cytidine deaminase or a functional
35   fragment thereof, one or more cofactors which enhance
the activity of cytidine deaminase.

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26. A method of treatment of the human or non-human
animal body to regulate cell proliferation, said method
comprising administering to said body one or more
cytidine deaminase inhibitors.

5

27. Compositions containing cytidine deaminase and
cofactors which enhance the activity of cytidine
deaminase as a combined preparation for simultaneous,
separate or sequential use in regulating cell
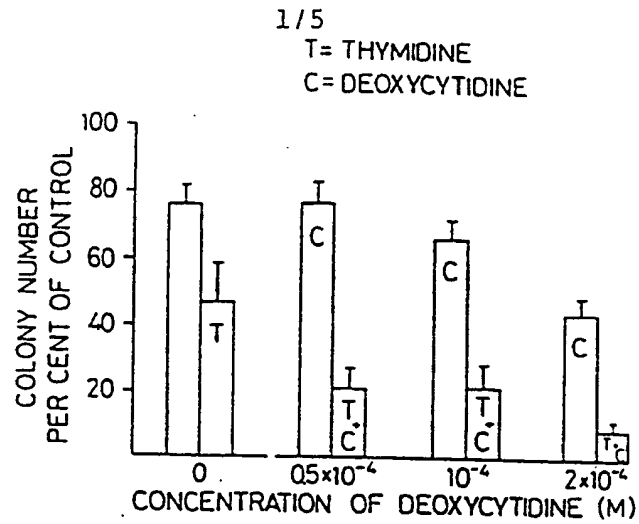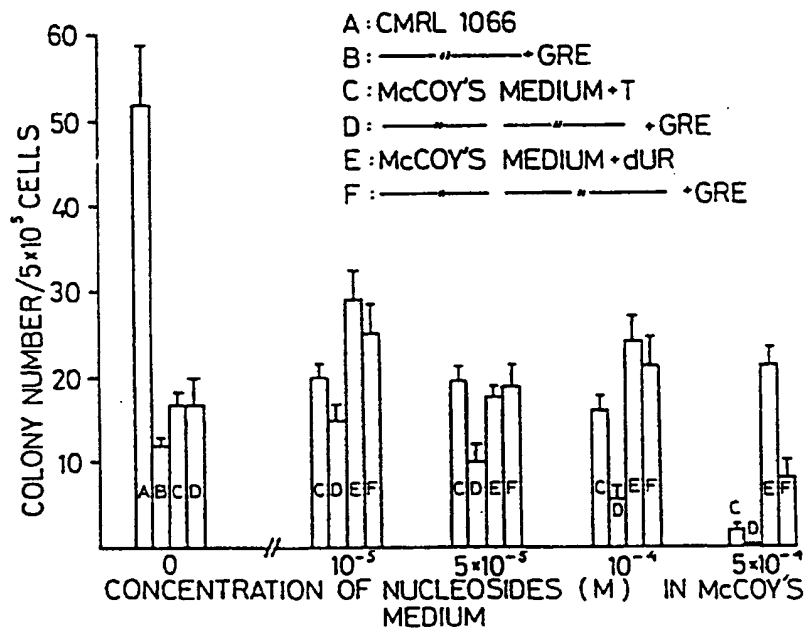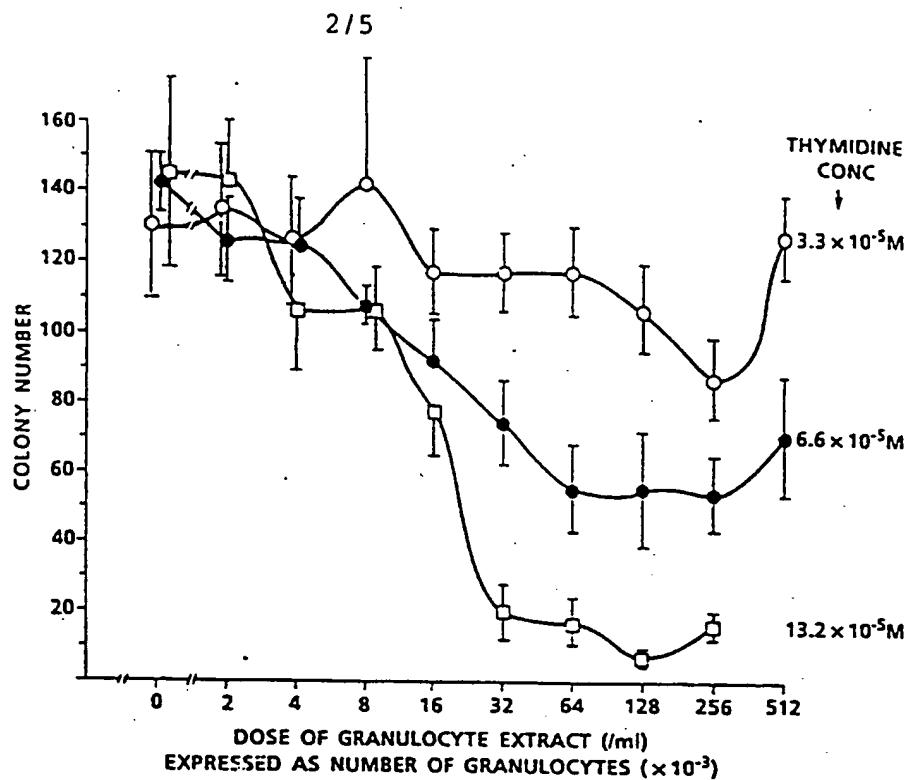
10 proliferation.

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T= THYMIDINE
C= DEOXYCYTIDINE

FIGURE 1

FIGURE 2

FIGURE 3

FIGURE 4

3 / 5
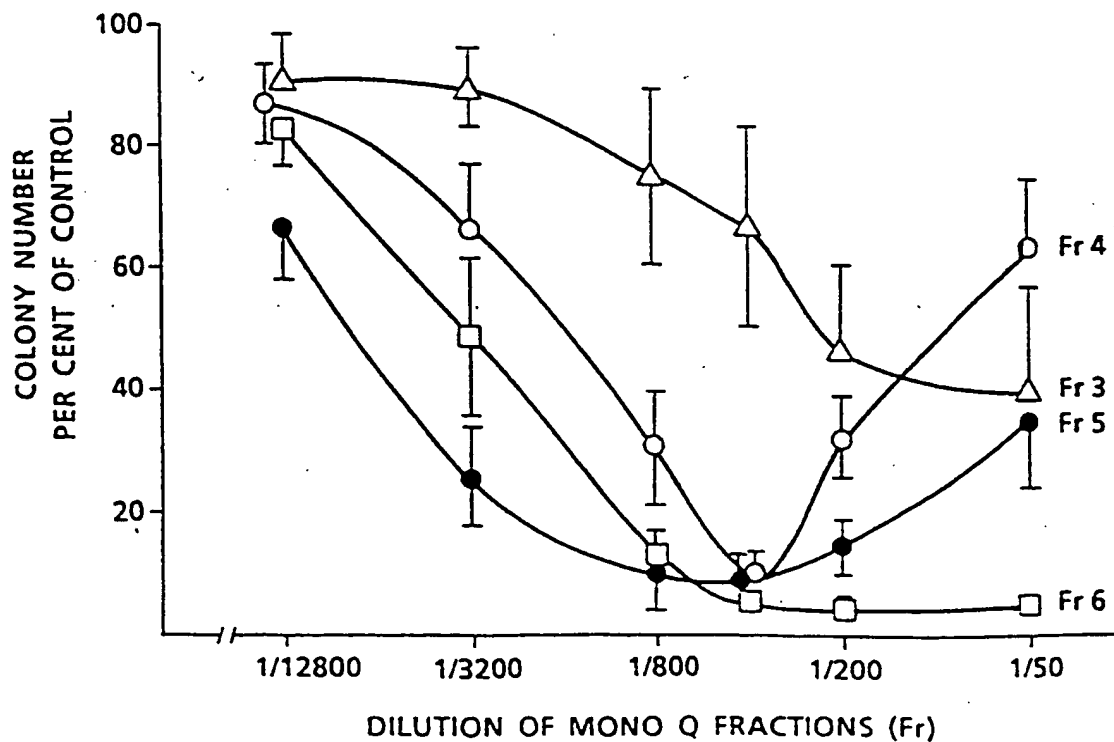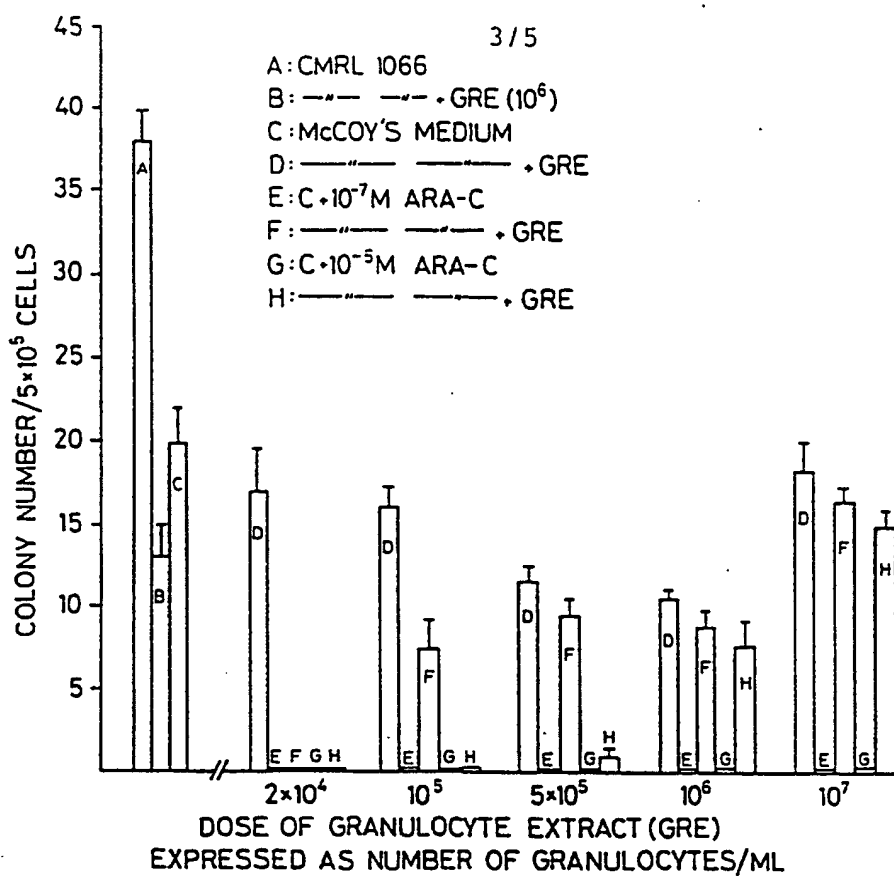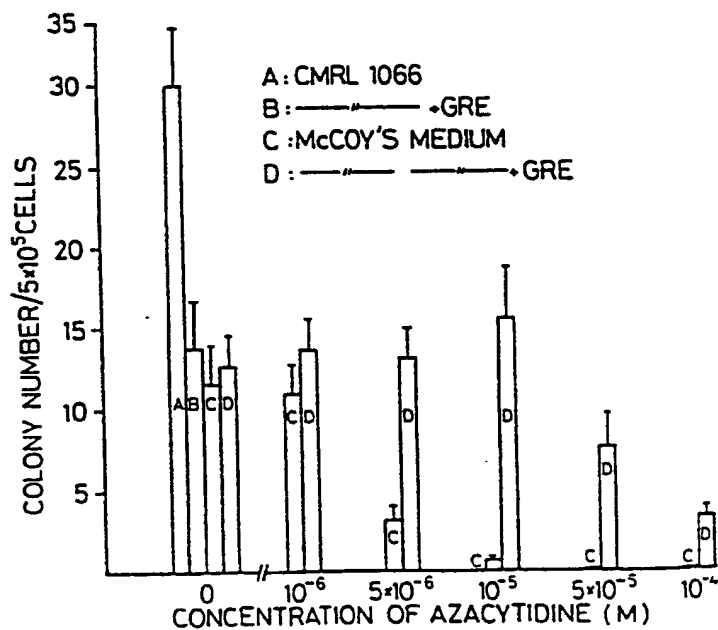
FIGURE 5

FIGURE 6

4 / 5
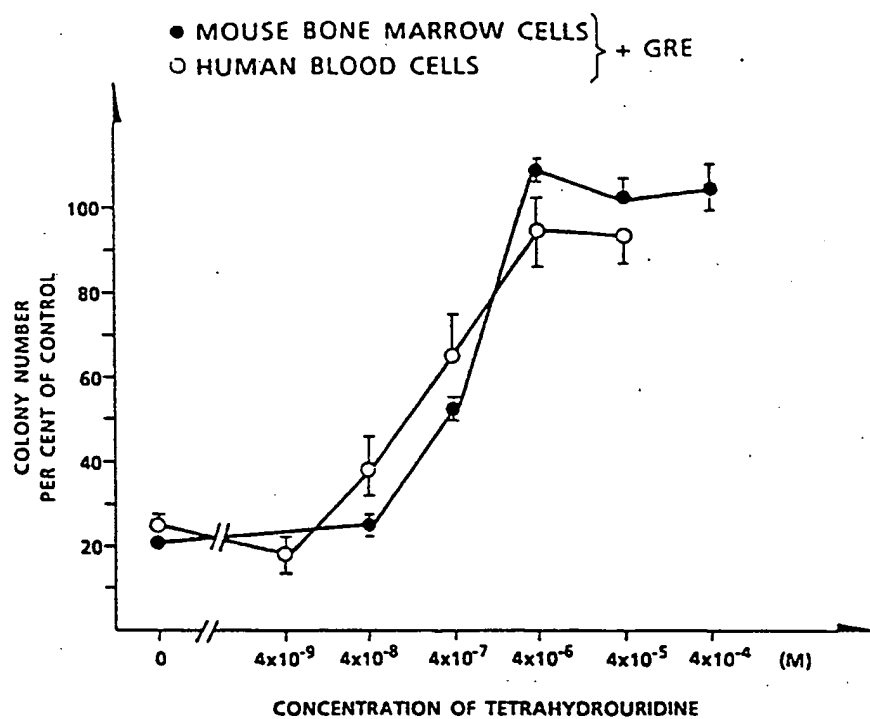
FIGURE 7
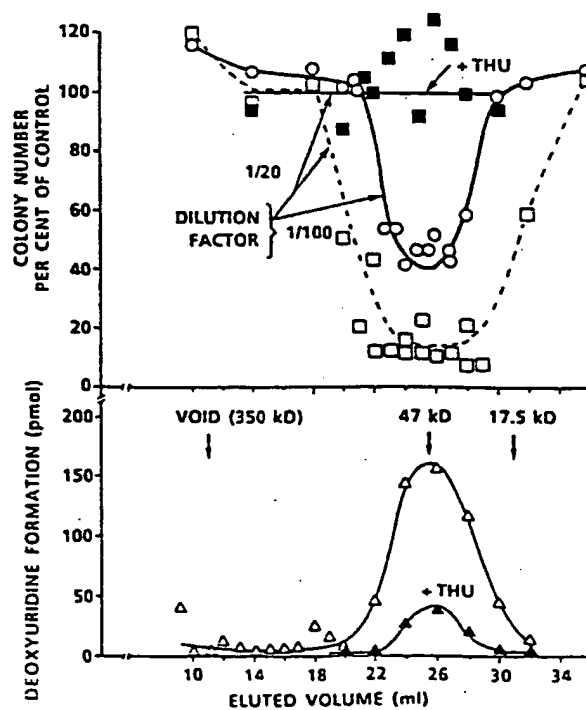
FIGURE 8
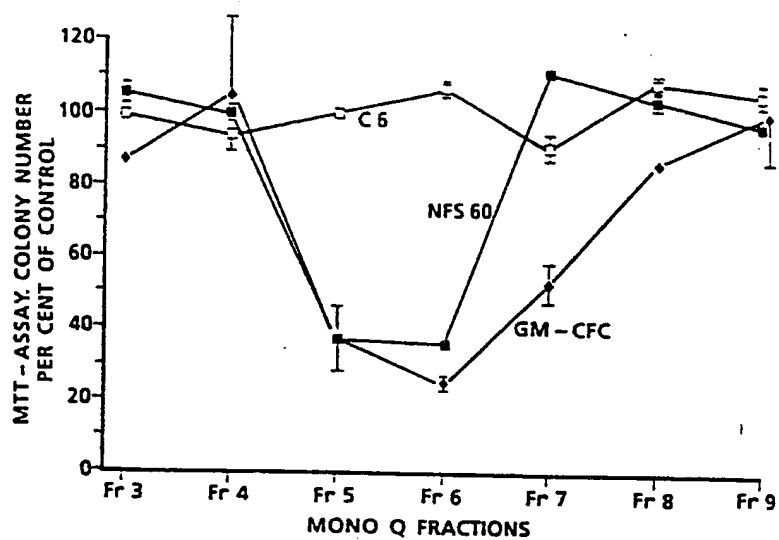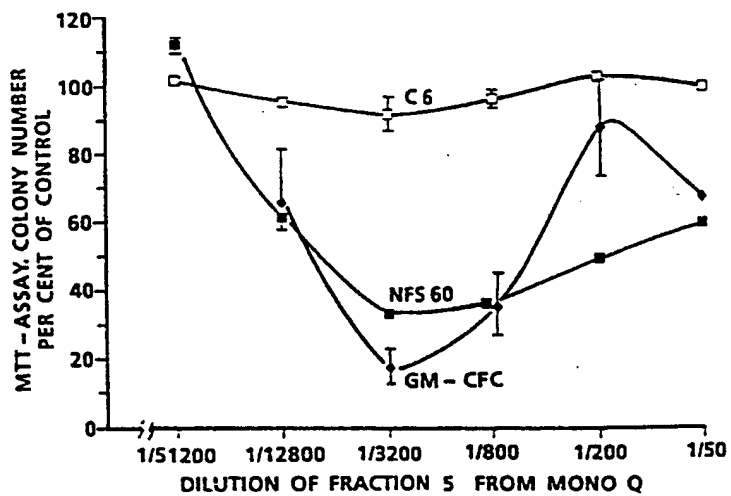
FIGURE 9

FIGURE 10

INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER
IPC 5   A61K37/54

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 5   C12N   A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	EXPERIMENTAL HEMATOLOGY vol. 22, no. 2 , February 1994 pages 208 - 214 ARNE BOYUM ET AL. 'IDENTIFICATION OF CYTIDINE DEAMINASE AS INHIBITOR OF GRANULOCYTE-MACROPHAGE COLONY FORMATION.' -----	1-27

☐ Further documents are listed in the continuation of box C.   ☐ Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
12 September 1994	I- 4 -10- 1994

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Rempp, G

| Box I | Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet) |

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

Remark : Although claims 24-26 are directed to a method of treatment of (diagnostic method practised on) the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

| Box II | Observations where unity of invention is lacking (Continuation of item 2 of first sheet) |

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.